IN THE CLAIMS:

Pleas cancel Claims 1-8 and 20, and amend Claims 16, 17, and 19, all without prejudice. The specific changes to the amended claims are shown on a separate set of pages attached hereto and entitled **VERSION WITH MARKINGS TO SHOW CHANGES MADE**, which follows the signature page of this Amendment. On this set of pages, the insertions are double underlined while the ~~deletions are struck through~~. A clean version of the entire set of pending claims is attached as a separate set of pages and entitled **CLEAN VERSION OF THE ENTIRE SET OF PENDING CLAIMS**.

REMARKS

The present invention is related to a procedure to block the replication of reverse transcriptase dependent viruses by the use of inhibitors of deoxynucleotides synthesis. Claims 9-19 are being examined in this application. Claims 1-8 and 20 have been canceled as being directed to a single compound. Claims 16, 17, and 19 have been amended to give the full names for all acronyms. The claims meet the written description and "how to make" and "how to use" enablement requirements of 35 USC § 112, first paragraph, as well as the definiteness requirement of 35 USC § 112, second paragraph, per Balzarini, J., "Effect of antimetabolite drugs ..." Pharmacol Ther 2000 Aug-Sep, 87(2-3):175-187 attached. Balzarini confirms that, in view of the combination of hydroxyurea (HU), a ribonucleotide reductase inhibitor, and 2',3'-dideoxyinosine (ddI), a nucleoside reverse transcriptase inhibitor (NRTI), as disclosed in the Lori et al. patents, it was obvious that this principle should be viable for the combination of other NRTIs (page 179, second column, first line of new paragraph), such as the NRTIs clinically approved or currently subjects of clinical trials for treatment of HIV: AZT (zidovudine), ddC (zalcitabine), d4T (stavudine), 3TC (lamivudine), ABC (abacavir), and 2'-F-dd-ara-A (lodenosine) (page 176, column 1, 3rd paragraph). It was also obvious that any modality that would deplete the intracellular pool of deoxyribonucleoside phosphates could substitute for HU (page 185, column 1, 1st paragraph). As for the disclosures of the Malley et al. patents which specifically exclude the combination of HU

and AZT as inactive against HIV in quiescent cells in culture, the disclosures of the Lori et al. patents specifically include the combination of HU and AZT as active against HIV in activated cells in culture. Before preparing a terminal disclaimer to obviate a double patenting rejection over USPs 5,521,161, 5,736,527, 6,046,175, and 6,194,390, Applicant respectfully brings USP 6,093,702 to the attention of the patent office for its consideration. Reexamination and reconsideration of the application, as amended, are respectfully requested.

CONCLUSION

In view of the above, it is submitted that the claims are in condition for allowance. Reconsideration and withdrawal of all outstanding rejections are respectfully requested. Allowance of the claims at an early date is solicited. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 11/13/01

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

On this set of pages, the insertions are double underlined while the ~~deletions are struck through~~.

9. (Pending) A method for inhibiting replication of reverse transcriptase dependent virus in animal cells, comprising the steps of administering to said cells a compound that depletes the intracellular pool of deoxyribonucleoside phosphate, in conjunction with administering to said cells an antiviral nucleoside phosphate analog.

10. (Pending) The method of claim 9, wherein said deoxynucleoside phosphate depleting compound is an inhibitor of ribonucleotide reductase.

11. (Pending) The method of claim 10, wherein said compound is hydroxyurea.

12. (Pending) A method for inhibiting replication of reverse transcriptase dependent viruses in animal cells, comprising the steps of administering to said cells a first compound that depletes the intracellular pool of deoxyribonucleoside phosphate, in conjunction with a second compound that serves to inhibit replication of said virus by terminating DNA chain elongation.

13. (Pending) The method claim 12, wherein said second compound inhibits replication by premature termination of viral DNA synthesis to produce incomplete viral DNA.

14. (Pending) The method of claim 12, wherein said first compound is an inhibitor of ribonucleotide reductase.

15. (Pending) The method of claim 14, wherein said first compound is hydroxyurea.

16. (Amended) The method of claim 15, wherein said second compound is selected from the group consisting of 2',3'-dideoxyinosine (ddI), 2',3'-dideoxycytidine (ddC), 2'-fluoro-2',3'-dideoxyarabinosyl adenosine (2'-F-dd-ara-A), 2'-fluoro-2',3'-dideoxyarabinosyl inosine (2'-F-dd-ara-I) and 2'-fluoro-2',3'-dideoxyarabinosyl guanosine (2'-F-dd-ara-G).

17. (Amended) The method of claim 12, wherein said second compound is selected from the group consisting of a dideoxynucleoside and 3'-azido-2',3'-dideoxythymidine (AZT).

18. (Pending) The method of claim 16, wherein said dideoxynucleoside is a 2'-fluoro purine dideoxynucleoside.

19. (Amended) The method of claim 16, wherein said dideoxynucleoside is selected from the group consisting of 2',3'-dideoxyinosine (ddI), 2',3'-dideoxycytidine (ddC), 2'-fluoro-2',3'-dideoxyarabinosyl adenosine (2'-F-dd-ara-A), 2'-fluoro-2',3'-dideoxyarabinosyl inosine (2'-F-dd-ara-I) and 2'-fluoro-2',3'-dideoxyarabinosyl guanosine (2'-F-dd-ara-G).

CLEAN VERSION OF ENTIRE SET OF PENDING CLAIMS

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19. (Amended) The method of claim 16, wherein said dideoxynucleoside is selected from the group consisting of 2',3'-dideoxyinosine (ddI), 2',3'-dideoxycytidine (ddC), 2'-fluoro-2',3'-dideoxyarabinosyl adenosine (2'-F-dd-ara-A), 2'-fluoro-2',3'-dideoxyarabinosyl inosine (2'-F-dd-ara-I) and 2'-fluoro-2',3'-dideoxyarabinosyl guanosine (2'-F-dd-ara-G).

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Pharmacology & Therapeutics 87 (2000) 175–187

Pharmacology & Therapeutics

Associate editor: D. Shugar

Effect of antimetabolite drugs of nucleotide metabolism on the anti-human immunodeficiency virus activity of nucleoside reverse transcriptase inhibitors

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Abstract

A number of attempts are currently underway to combine antimetabolite drugs of nucleotide metabolism with a nucleoside reverse transcriptase inhibitor (NRTI) targeting human immunodeficiency virus (HIV) to improve the antiviral efficacy of the NRTIs and to better control HIV drug resistance. Hydroxyurea, a ribonucleotide reductase inhibitor, is currently combined with the NRTI didanosine (2′,3′-dideoxyinosine) in clinical trials. However, other cellular target enzymes, including thymidylate synthase, inosinate dehydrogenase, cytidine-5′-triphosphate synthetase, and other enzymes from the de novo nucleotide biosynthesis pathway, can also be considered to potentiate the antiviral action of NRTIs. The underlying reasons for the potentiation of the antiviral activity of the NRTIs by antimetabolite drugs of nucleotide metabolism can be multiple. Decreased endogenous 2′-deoxynucleoside-5′-triphosphate (dNTP) pools result in a better competition of the NRTI (as its triphosphate derivative), with the dNTPs for the virus-encoded reverse transcriptase to be recognized as a substrate for the DNA polymerization reaction and subsequently to be incorporated into the growing viral DNA chain. Also, an increased metabolism (phosphorylation) of the NRTI by stimulatory enzyme feedback mechanisms may result in the production of higher levels of NRTI triphosphate. Thus, higher intracellular ratios of NRTI-triphosphate/dNTP created by well-defined combinations of NRTIs and antimetabolite drugs enable a more profound inhibitory effect of the NRTI against the reverse transcriptase (and thus, against the virus) and a better suppression of resistant (mutant) virus strains. A profound evaluation of this relatively new concept in the clinical setting will reveal whether this approach will establish a place in future treatment modalities of HIV infections. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Nucleoside reverse transcriptase inhibitors (NRTIs); Antimetabolite drugs; Ribonucleotide reductase; Inosinate dehydrogenase; Thymidylate synthase; HIV; AIDS

Abbreviations ABC, carbocyclic 2′,3′-didehydro-2′,3′-dideoxy-2-amino-6-cyclopropylaminopurine riboside, abacavir; ANP, acyclic nucleoside phosphonate; AzdCyd, 2′-azido-2′-deoxycytidine; AzddDAPR, 3′-azido-2′,3′-dideoxy-2,6-diaminopurine riboside; AZT, 3′-azido-2′,3′-dideoxythymidine; AZTTP, 3′-azido-3′-deoxythymidine triphosphate; CBV, carbovir; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine, stavudine; d4TTP, 3′-deoxy-2′,3′-didehydrothymidine 5′-triphosphate; ddATP, 2′,3′-dideoxyadenosine-5′-triphosphate, ddC, 2′,3′-dideoxycytidine; ddCTP, dideoxycytidine-5′-triphosphate; ddDAPR, 2′,3′-dideoxy-2,6-diaminopurine riboside; ddI, 2′,3′-dideoxyinosine; ddIMP, dideoxyinosine monophosphate; ddNTP, 2′,3′-dideoxynucleoside-5′-triphosphate; dNTP, 2′-deoxynucleoside-5′-triphosphate; dThd, deoxythymidine; FddaraA, 9-β-D-(2′-fluoro-2′,3′-dideoxyarabinosyl)adenine; 5-FdUrd, 5-fluoro-2′-deoxyuridine; (−)FTC, 5-fluoro derivative of L-3′-thia-2′,3′-dideoxycytidine; 5-FU, 5-fluorouracil; HIV, human immunodeficiency virus, HU, hydroxyurea; IMP, inosinate; IMP-D, inosinate dehydrogenase; MPA, mycophenolic acid; MTX, methotrexate; NRTI, nucleoside reverse transcriptase inhibitor; PALA, *N*-(phosphonoacetyl)-L-aspartate; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PMEA, 9-(2-phosphonylmethoxyethyl)adenine, adefovir; PMPA, 9-(2-phosphonylmethoxypropyl)adenine, tenofovir; Rib-MP, 5′-monophosphate derivative of ribavirin; RR, ribonucleotide reductase; RT, reverse transcriptase, 3TC, L-3′-thia-2′,3′-dideoxycytidine; TK, thymidine kinase; TS, thymidylate synthase; XMP, xanthosine monophosphate.

Contents

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PII: S0163-7258(00)00050-4

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1. Introduction

Human immunodeficiency virus (HIV) does not encode for nucleoside or nucleotide-metabolizing enzymes (i.e., nucleoside kinases, ribonucleotide reductase [RR], thymidylate synthase [TS]), and, therefore, entirely depends on the metabolic machinery of the host cells for its DNA and RNA synthesis. However, besides a number of structural and regulatory proteins, HIV also encodes several functional proteins (enzymes) that are essential for viral protein cleavage (protease), for DNA synthesis (reverse transcriptase, RT) and for incorporation of the viral DNA product into the cellular genome (integrase). Given the fact that HIV efficiently replicates in human cells, including lymphocytes and monocytes/macrophages, a considerable and continuous 2′-deoxynucleoside-5′-triphosphate (dNTP) supply is required to keep the intracellular replication of HIV ongoing.

The HIV-encoded RT is a DNA polymerase that contains at least three different functions in one enzyme: (1) an RNA-dependent DNA polymerase activity, converting the single-stranded viral RNA genome to an RNA/DNA hybrid; (2) an RNAseH activity, hydrolyzing (removing) the original RNA strand from the RNA/DNA hybrid; and (3) a DNA-dependent DNA polymerase activity, converting the remaining single-stranded DNA to a double-stranded DNA.

To date, six nucleoside analogues targeted at HIV RT are clinically approved for treatment of HIV-infected individuals. They are 3′-azido-2′,3′-dideoxythymidine (AZT, zidovudine), 2′,3′-dideoxycytidine (ddC, zalcitabine), 2′,3′-dideoxyinosine (ddI, didanosine), 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T, stavudine), L-3′-thia-ddC (3TC, lamivudine), and carbocyclic 2′,3′-didehydro-2′,3′-dideoxy-2-amino-6-cyclopropylaminopurine riboside (ABC, abacavir) (Fig. 1) (Balzarini & De Clercq, 1998; De Clercq, 1998a, 1998b). In addition, a number of other nucleoside reverse transcriptase inhibitors (NRTIs), including the 5-fluoro derivative of 3TC [(−)FTC, emtricitabine] and 9-β-D-(2′-fluoro-2′,3′-dideoxyarabinosyl) adenine (FddaraA, lodenosine), are currently subjects of clinical Phase II/III trials (Fig. 1). Also, two members of another structural class of nucleotide analogues, viz. acyclic nucleoside phosphonates (ANPs) (i.e., 9-(2-phosphonylmethoxyethyl)adenine [PMEA, adefovir] and 9-(2-phosphonylmethoxypropyl)adenine [PMPA, tenofovir]) (De Clercq, 1998a, 1998b), are currently subjects of Phase II/III clinical trials for the treatment of HIV (for an overview of NRTIs and ANPs applied for HIV therapy, see Kinchington et al., 1998; De Clercq, 1998a, 1998b) (Fig. 2). The NRTIs need to be converted to their 5′-triphosphate derivatives before they become inhibitory against HIV replication. As 5′-triphosphates, the NRTIs compete with the natural nucleotides (i.e., dTTP, dCTP, dGTP, dATP) for recognition by the virus-encoded RT as substrates to become incorporated into the growing DNA chain (Fig. 3). Similarly, the ANPs need to be converted to their diphosphate metabolites before they can compete with the endogenous dNTPs for recognition by HIV RT and subsequent incorporation into the viral DNA. Incorporation of the NRTIs and ANPs into the growing viral DNA leads to DNA chain termination since the NRTIs and ANPs lack a free hydroxyl function, required for further elongation of the DNA chain. The latter property (DNA chain termination) of the NRTIs and ANPs has been considered as crucial in the eventual inhibition of virus replication (Balzarini & De Clercq, 1996).

Due to the fact that NRTIs and ANPs need to be metabolized to the 5′-triphosphate or -diphosphate derivatives, respectively, to become antivirally active, and need to compete with the endogenous dNTP pools for substrate recognition and eventual incorporation by HIV-1 RT, any changes in the metabolism of the natural nucleotides and of the NRTIs/ANPs, including changes of endogenous dNTP pools, will affect the efficiency of conversion of the NRTIs and ANPs to their phosphorylated derivatives, and their potential to compete with the endogenous dNTP pools for recognition by the RT. Thus, it is obvious that antimetabolite drugs, which affect nucleotide pool levels or suppress or activate nucleotide-metabolizing enzymes, may influence the eventual antiviral efficacy of the NRTIs and ANPs.

2. Effect of restriction of endogenous 2′-deoxynucleoside-5′-triphosphate pools on human immunodeficiency virus replication

It has been observed that in quiescent peripheral blood lymphocytes (PBLs) viral (HIV) replication can be easily initiated, but is terminated prematurely. As a consequence, no HIV progeny are released by quiescent cells. In contrast, a pool of unintegrated viral DNA is observed under these conditions, remaining latent. Stimulation of these cells, however, results in an instant increase of the processivity of viral DNA synthesis and in the synthesis of proviral DNA that gets incorporated into cellular DNA, leading to release of viral progeny (Zack et al., 1990; Stevenson et al., 1990).

The extremely slow and inefficient formation of an early viral DNA pool in quiescent PBLs is due to the remarkably low dNTP pool levels in these cells. Stimulation of HIV-infected PBLs results not only in a marked increase of the

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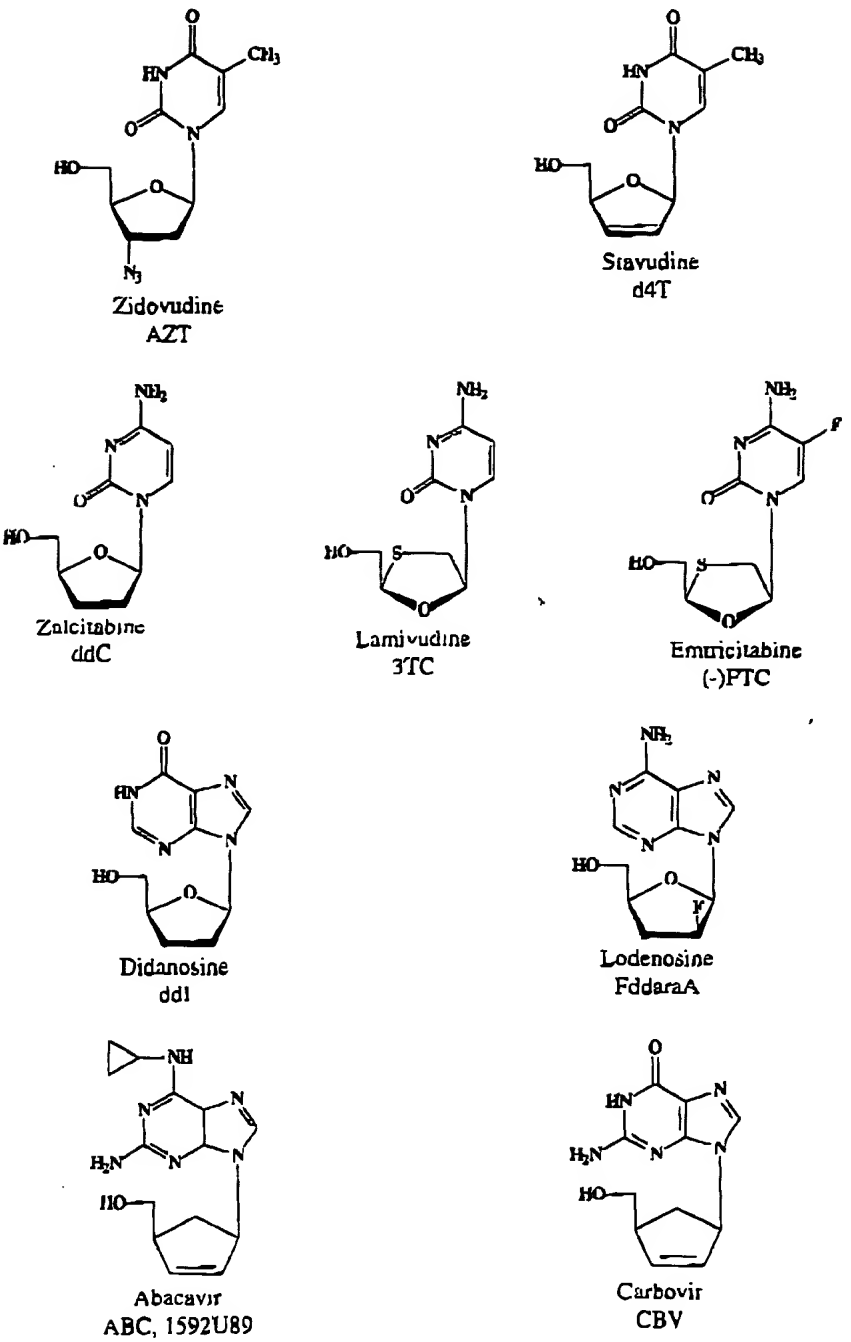

Fig. 1 Structural formulae of NRTIs.

RT-catalyzed HIV DNA formation and replication, but also in substantially higher intracellular dNTP pools. Thus, HIV replication seems to be highly dependent on the intracellular concentration of dNTPs, and the relatively low endogenous dNTP concentrations in resting T lymphocytes may preclude, if not restrict, HIV replication in this type of cell. Gao et al. (1993) indeed could demonstrate that HIV-1 RT activity is influenced by the dNTP substrate concentration. When the dNTPs in the RT reaction mixture were used at those concentrations that are known to be present in quiescent PBLs, a very low RT activity was noted. However, when the dNTP levels were increased by 10-fold for dATP (~3.7 μM), dGTP (~8.5 μM), and dCTP (~23 μM) and by 5-fold for dTTP (~30 μM) (i.e., at concentrations that were found in phytohemagglutinin [PHA]-stimulated lymphocytes), the RT reaction was dramatically speeded up, resulting in the

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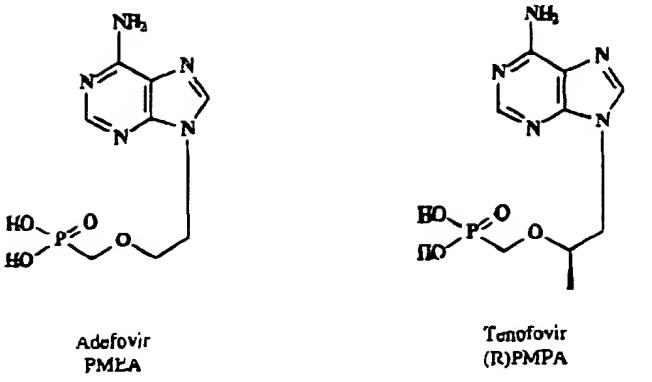

Fig. 2. Structural formulae of ANPs.

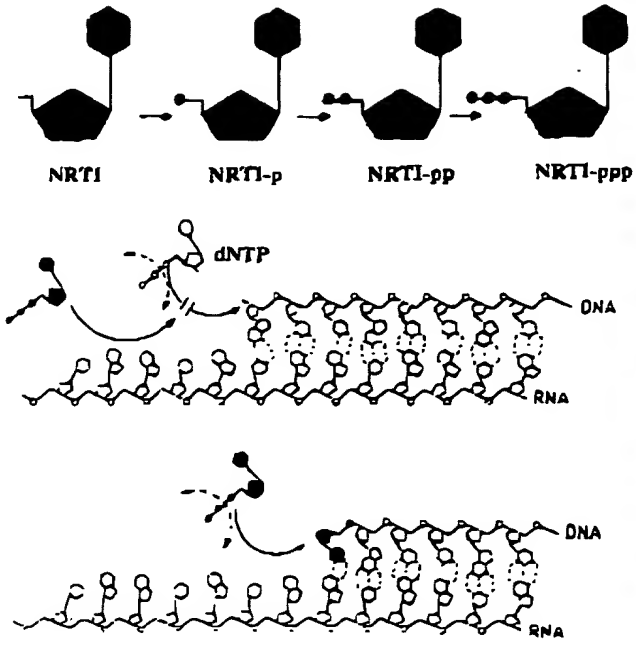

Fig. 3. Metabolism and DNA chain termination by NRTIs.

rapid formation of full-length viral DNA (Gao et al., 1993). Interestingly, hydroxyurea (HU) exposed to stimulated PBLs decreased the dNTP levels and the rate of viral DNA synthesis to levels comparable with those observed in quiescent PBLs. These observations indicated that pharmacological induction of low dNTP levels may represent a therapeutic approach for inhibition of HIV-1 replication.

Based on this promise, attempts have been made to lower one or several dNTP pools in order to enhance the antiviral (anti-HIV) effect. Such selective depletion of dNTP pools can be afforded by HU, a well-known inhibitor of RR that catalyses the conversion of nucleoside-5′-diphosphates to 2′-deoxynucleoside-5′-diphosphates. HU interacts with RR, and may result in a decrease of dNTPs (Bianchi et al., 1986), most pronounced for dATP, followed by dCTP and dGTP. HU usually has the poorest effect on dTTP levels in PBLs (Collins & Oates, 1987; Slabaugh et al., 1991). HU has been shown to block HIV-1 replication in acutely infected primary human lymphocytes (both quiescent and activated) and macrophages, as well as in blood cells infected in vivo obtained from individuals with acquired immunodeficiency syndrome (Lori et al., 1994). The antiviral effect in cell culture was achieved at nontoxic doses of HU that were lower than those currently used in human therapy. As already mentioned, HU (1 mM) exposure to stimulated PBLs resulted in a substantial depletion of the dNTP pools and concomitant reduction of the rate of HIV-1 DNA synthesis. Thus, HU retarded the completion of viral DNA synthesis in PHA-stimulated PBLs, resulting in a pattern of DNA synthesis inhibition that was quite similar to that observed in quiescent PBLs (Gao et al., 1993). Also, Meyerhans et al. (1994) demonstrated that HIV-1 replication in PBLs was almost completely abolished by 3 mM HU, and that this effect could be readily reversed by the addition of 2′-deoxyadenosine, known to reverse dNTP pool changes mediated by HU (Slabaugh et al., 1991).

Another indication that modification of dNTP pools could afford an antiviral effect in cell culture was provided by the observation that addition of 10–50 μM deoxythymidine (dThd) to established cell lines (i.e., H9, U937, CEM) resulted in a dramatic reduction of HIV-1 production (Meyerhans et al., 1994). This effect could be substantially reversed by addition of deoxycytidine (dCyd). These data are consistent with the idea that dThd modulated HIV-1 replication by influencing dNTP pool levels, in particular by reducing the dCTP pools, most likely due to inhibition of the RR-catalyzed CDP reduction to dCDP by dTTP.

HU has been widely used over the last 30 years with some success for the treatment of human malignancies, especially chronic myelogenous leukemia and other myeloproliferative syndromes (Belt et al., 1980; Kennedy, 1992; Donehower, 1992). Recently, HU has also been used in the management of sickle cell anemia in adults (Charache, 1997). Plasma peak levels of 0.5–2.5 mM and through concentrations of 0.2–0.5 mM could be achieved by oral administration. The major side effect of HU includes myelosuppression (i.e., granulocytopenia) (Timson, 1969). However, by interfering with lymphocyte proliferation, HU may also restrict some important immune responses. To assess the efficacy of the therapeutic use of HU for the treatment of HIV-1, a number of HIV-1-infected patients have been subjected to monotherapy with HU for 8–19 weeks. In none of the patients were the immunologic, hematologic, and quantitative virologic parameters, including plasma viremia and number of provirus-containing cells, modified during the course of therapy. The number of $CD4^+$ cells tended to further decline. Thus, although this study embraced a limited number of patients, no signs of improvement in HIV disease and therapeutic benefit were detected (Giacca et al., 1996b). A similar study performed by others reached a similar conclusion (Simonelli et al., 1996).

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. Effect of ribonucleotide reductase inhibition on the nti-human immunodeficiency virus activity of ucleoside reverse transcriptase inhibitors

Since it has been recognized that the anti-HIV-1 activity f NRTIs does not depend mainly on the absolute levels of the ',3'-dideoxynucleoside-5'-triphosphates (ddNTPs) generated ntracellularly, but rather on the ratio of the intracellular oncentration of ddNTP/dNTP (Gao et al., 1994), HU and elated RR inhibitors may be beneficial when combined vith NRTIs. Thus, decreased dNTP levels may favor the ompetitive advantage of the ddNTP against HIV-1 RT, reulting in an increased antiviral efficacy of the NRTI. Morever, in certain cases, antimetabolite drugs affecting the dNTP ool levels may also concomitantly stimulate some enzymes nvolved in nucleotide biosynthesis and thus, eventually further peed up ddNTP synthesis. Therefore, it would be of particular nterest to combine antimetabolites with NRTIs.

The best known example based on this principle is the ombination of HU with ddI (see Johns & Gao, 1998). It is nown that HU depletes dATP pools more severely than ther dNTP pools (Collins & Oates, 1987; Slabaugh et al., 1991; Gao et al., 1998). Therefore, in vitro studies of HU ombinations with NRTIs, in particular ddI, have been performed (Fig. 4). Combination of HU (0.1 mM) with the NRTI ddI generated a synergistic inhibitory effect without increasing toxicity in activated peripheral blood mononuclear cells (PBMC) (derived from an HIV-1-infected individual) (Lori et al., 1994; Foli et al., 1997). HU concentrations as low as 0.1 mM were also found to time-dependently potentiate the antiviral effect of ddI by at least 6-fold in activated PBMC experimentally infected with HIV-1. Indeed, combinations of HU and ddI were shown to result in total suppression of virus production in resting HIV-1-infected lymphocytes, and had no effect on the cell's ability to replicate normally after treatment (Malley et al., 1994). Thus, in all of these studies, it was shown that HU was effective as a potentiating agent, in combination with ddI, at much lower concentrations than when used alone. This fact may be of clinical significance in drug-treated patients.

Once the principle had been proven that the RR inhibitor HU enhances the antiviral efficacy of ddI in cell culture, due to depletion of the endogenous dATP pools, it seemed obvious that this principle should also be viable for other NRTIs that competes with dATP pools at the level of HIV-1 RT. Gao et al. (1995) indeed have been able to show that HU enhanced the anti-HIV potency of 2'-β-fluoro-2',3'-dideoxyadenosine (FddaraA, lodenosine) in PBL (Fig. 4). In fact, the potentiation of the antiviral efficacy of FddaraA by HU was more pronounced than with ddI, allowing a 7.1-fold reduction in the optimal dose of FddaraA in PHA-activated HIV-1-infected PBLs in the presence of 0.1 mM HU. Also, Palmer et al. (1999) recently demonstrated that HU potentiated the in vitro anti-HIV-1 activity of PMEA and PMPA against a wild-type laboratory HIV-1 strain, and also against a panel of 5 well-characterized drug-resistant HIV isolates (Fig. 4). HU (50 μM) proved sufficient to afford a significant potentiating effect against the virus, the extent of which depended highly on the nature of the mutant virus strain (ranging from 7- to 61-fold for PMPA and from 4- to 134-fold for PMEA).

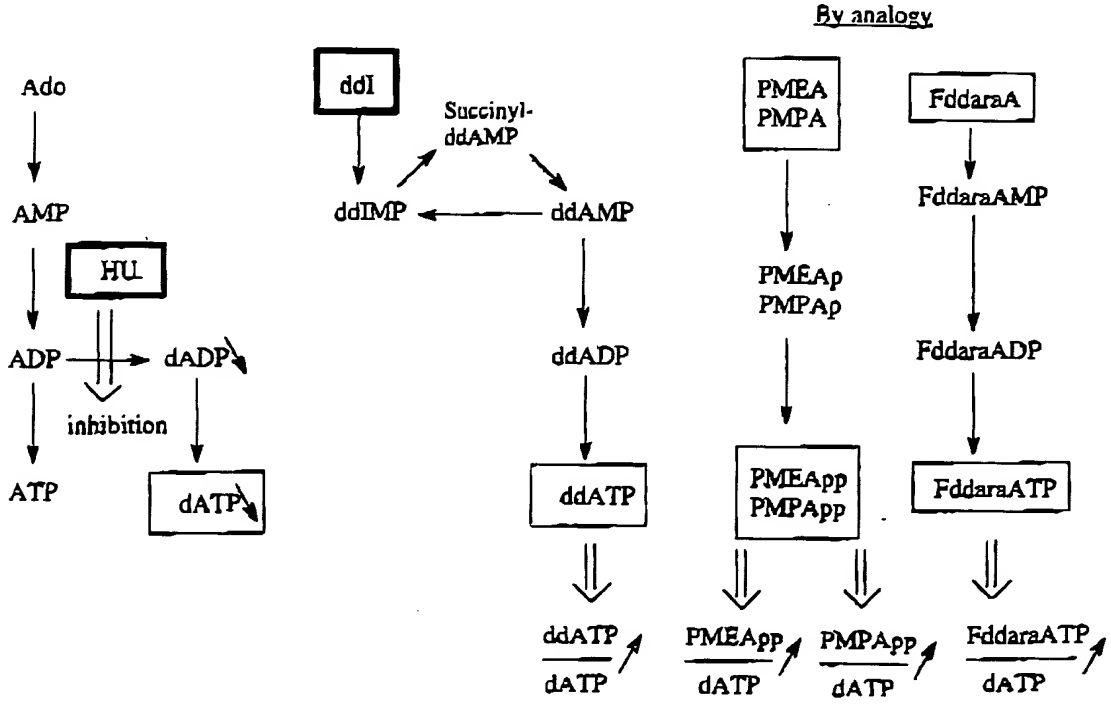

Fig. 4 Combination of HU with purine NRTIs and purine ANPs

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RR inhibitors have also been combined with NRTIs other than ddI. In fact, as early as 1987, Balzarini et al. reported that high-dose dThd severely decreased endogenous dCTP pools (presumably due to inhibition of RR-catalysed CDP reduction by dTTP), resulting in a stimulation of zalcitabine (ddC) metabolism to its dideoxycytidine-5′-triphosphate (ddCTP) derivative (Fig. 5). Thus, in addition to decreasing the dCTP pools, the ddCTP levels were concomitantly increased, resulting in much higher ddCTP/dCTP ratios than when ddC was administered without dThd. Combination of ddC and dThd in HIV-1-infected human ATH8 cells afforded an increased antiviral effect without increasing toxicity in cell culture. In the same study, it was shown that 50 μM HU was also able to deplete dCTP pools and to increase ddCTP formation by 4-fold compared with control cultures without HU (Balzarini et al., 1987). However, no antiviral data on the combination of HU with ddC were provided in this study. The concomitant increase of ddCTP formation could be explained by at least two different phenomena. First, dTTP is known to be an activator of dCyd kinase (Maley & Maley, 1962; Durham & Ives, 1970), and thus, high intracellular dTTP levels may result in increased ddC phosphorylation by the activated dCyd kinase. Second, since dCTP is a feed-back inhibitor of dCyd kinase (Maley & Maley, 1962; Durham & Ives, 1970), it is expected that lowering the intracellular dCTP levels may have a stimulatory effect on dCyd kinase, resulting in an increased phosphorylation of ddC. Thus, combination of dThd (or HU) with the NRTI ddC is an example where not only a selective decrease of one of the endogenous dNTP pools increases the competitive advantage of the NRTI-triphosphate against HIV-1 RT, but also where the metabolism of the NRTI to its triphosphate is enhanced, further contributing to a high and pronounced ddNTP/dNTP ratio shift (Fig. 5).

Karlsson et al. (1989) showed that HU increases the phosphorylation of the pyrimidine NRTI AZT and another thymidine analogue (3′-fluoro-2′,3′-dideoxythymidine) in CEM cell cultures. Later, Bianchi, Reichard and co-workers also reported that RR inhibitors, such as 2′-azido-2′-deoxycytidine (AzdCyd), gemcitabine (2′,2′-difluorocytidine), and HU (Fig. 6), increase the conversion of AZT to its 5′-triphosphate derivative. (Bianchi et al., 1994; Giacca et al., 1996a). These investigators could demonstrate that increased phosphorylation of AZT in CEM cells was caused by a prolongation of the S phase of the cell cycle, afforded by these drugs (upon inhibition of RR by the 5′-diphosphate derivative of AzdCyd and gemcitabine, or directly by HU). Indeed, since AZT is phosphorylated by cytosolic thymidine kinase (TK)-1, a cell-cycle-regulated enzyme with highest activity in late G1 and S phases, the AZT phosphorylation presumably was increased by the higher enzymatic activity of TK-1 in the cell cultures that were exposed to the RR-inhibitors. HU, in combination with AZT, was proven to potentiate the antiviral efficacy of AZT in both CEM cells and PBMC (Palmer & Cox, 1997).

4. Effect of thymidylate synthase inhibitors on the anti-human immunodeficiency virus activity of nucleoside reverse transcriptase inhibitors

TS is a key enzyme of thymidylate synthesis and the only de novo enzyme that is responsible for providing thymine nucleotides required for DNA synthesis (Santi, 1980, 1981).

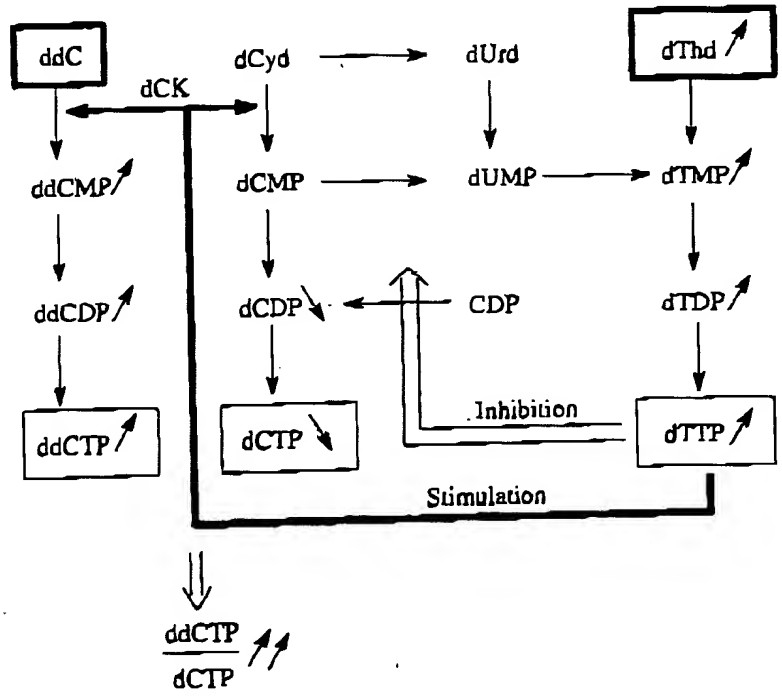

Fig 5 Combination of dThd with the pyrimidine NRTI ddC. The lowered dATP pools by HU result in a higher ratio of ddATP/dATP.

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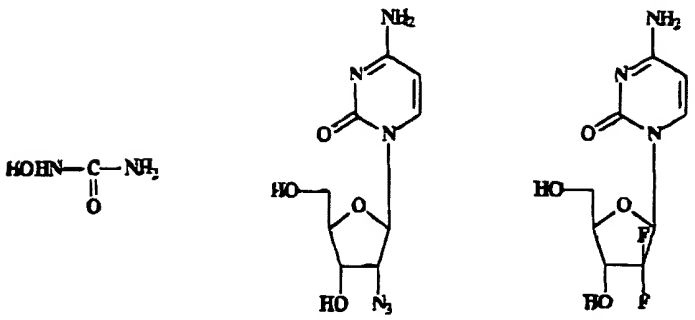

Fig. 6. Structural formulae of the ribonucleotide reductase inhibitors HU, AzdCyd, and gemcitabine. The closed black arrow represents a stimulatory effect of increased dTTP pools on 2'-deoxycytidine kinase (dCK). The open double arrow represents an inhibitory effect of dTTP on RR (conversion CDP to dCDP). Consequently, the lowered dCTP and increased ddCTP pools result in a higher ratio of ddCTP/dCTP.

Administration of TS inhibitors seriously compromises dTTP synthesis, resulting in decreased dTTP levels. Therefore, combination of TS inhibitors with NRTIs, in particular thymine NRTI derivatives, such as AZT and d4T, would be expected to increase the competitive advantage of the NRTI-triphosphate with the endogenous (lowered) dTTP pools. Moreover, as also shown for the combination of RR inhibitors and ddCyd (Section 3), decreased dTTP pools will stimulate dThd kinase activity due to alleviation of the feedback inhibition of dThd kinase by the end product dTTP of this metabolic pathway (Bresnick & Karjala, 1964). This may lead to a pronounced increase of the intracellular ratio of NRTI-triphosphate/dTTP (Fig. 7). Ahluwalia et al. (1996) indeed have shown that agents that inhibit de novo pyrimidine nucleotide biosynthesis have the ability to increase d4T phosphorylation. Methotrexate and 5-fluoro-2'-deoxyuridine (5-FdUrd; Fig. 8) were amongst the most effective agents to afford this effect when combined with d4T in cell culture. Methotrexate (MTX) afforded ~6-fold higher 3'-deoxy-2',3'-didehydrothymidine 5'-triphosphate (d4TTP) levels at 1 and 5 μM, and 5-FdUrd afforded 8- to 10-fold higher d4TTP levels at 0.25 and 1 μM in human Molt cells. When PHA-stimulated PBMC were infected with HIV-1 and subsequently exposed to combinations of d4T and 5-FdUrd (0.2 μM), an 8-fold decrease in the d4T concentration could be afforded to attain a 50% reduction of HIV-1 p24 antigen production. At this 5-FdUrd concentration (0.2 μM), no significant anti-HIV effect of 5-FdUrd was observed when administered as a single drug. Thus, in view of the pharmacological properties of MTX and 5-FdUrd in cancer chemotherapy, combination of d4T with these agents at a low dose may have a potential application for potentiating the anti-HIV effect of d4T.

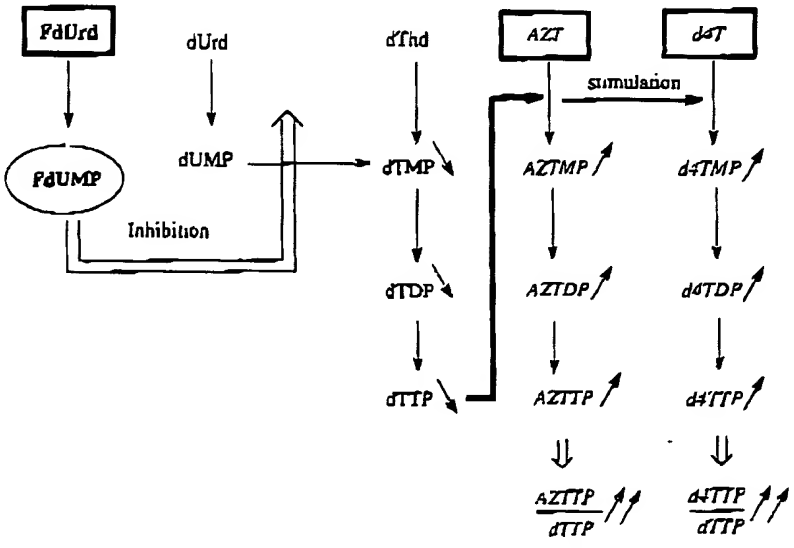

Fig. 7. Combination of a thymidylate synthase inhibitor (FdUrd) with pyrimidine NRTIs: AZT or d4T. The closed black arrow represents a stimulatory effect of decreased dTTP pools on TK. The open double arrow represents an inhibitory effect of FdUMP on thymidylate synthase (conversion of dUMP to dTMP). Consequently, the lowered dTTP pools and increased AZTTP and d4TTP pools result in a higher ratio of AZTTP/dTTP and d4TTP/dTTP.

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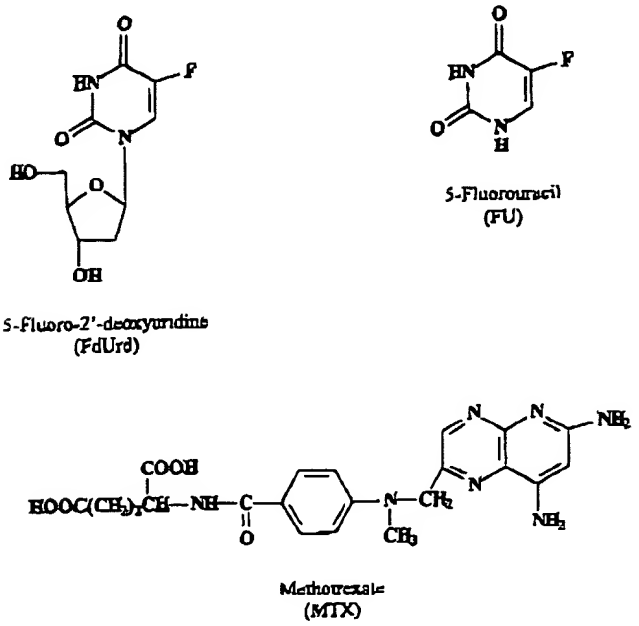

Fig. 8. Structural formulae of MTX, 5- FU, and 5- FdUrd.

The RR inhibitor HU, theoretically also lowering the dTTP pools, may afford similar effects on d4T phosphorylation. However, it is well known that HU can have varying effects on these dNTP pool levels, depending on the nature of the cell type and concentration of the drug used. HU has even been shown to increase dTTP (and dCTP pools) under certain experimental conditions (Gao et al., 1993; Bianchi et al., 1986). Therefore, it may not necessarily be beneficial to combine thymine NRTIs with HU.

In a later, and very recent, study, TS inhibitors (such as 5-fluorouracil [5-FU] and 5-FdUrd) have also been combined with AZT and d4T against wild-type HIV-1 and multidrug-resistant HIV-1 variants (Gao et al., 1999). In all cases, 1 μM 5-FU (and 0.2 μM 5-FdUrd) were able to markedly potentiate d4T and AZT inhibition of both wild-type and multi-NRTI drug-resistant virus strains in PBMC. In these studies, it could also be shown that dTTP levels dose-dependently decreased to 24% of control at increasing 5-FdUrd concentrations (0.05–0.80 μM), whereas d4TTP formation increased up to 6-fold in the presence of 0.80 μM 5-FdUrd. Consequently, the d4TTP/dTTP ratios increased to 9-fold in the presence of 0.05 μM 5-FdUrd, over 15-fold in the presence of 0.20 μM 5-FdUrd, and to 26-fold in the presence of 0.80 μM 5-FdUrd (Gao et al., 1999).

It is interesting and important to emphasize that such drug combinations also proved able to suppress the replication of NRTI-resistant HIV-1 strains. NRTI drug resistance appears to be predominantly associated with amino acid substitutions in the HIV RT. These mutations in the RT usually decrease RT affinities for the corresponding ddNTPs. The RT enzymes of the mutant viruses usually do not show marked changes in the k_{cat}/K_m ratios for the physiological dNTPs (Ueno et al., 1995; Martin et al., 1993). Therefore, partial or even complete restoration of the sensitivity of drug-resistant HIV-1 mutants to the ddNTPs can be achieved by lowering the level of the corresponding physiological nucleotide by pharmacological intervention. In the case of relatively modest K_i changes (i.e., 5- to 10-fold), sensitivity of the drug-resistant virus strains can be fully restored upon combination of these drugs with antimetabolites such as 5-FU. A similar phenomenon was observed previously by Lori et al. (1997) for HU and ddI.

5. Inosinate dehydrogenase inhibitors as potential candidate drugs to be combined with purine nucleoside reverse transcriptase inhibitors

Among the most best-known inhibitors of inosinate dehydrogenase (IMP-D) are ribavirin and mycophenolic acid (Fig. 9). Ribavirin is a broad-spectrum antiviral agent that has shown clinical efficacy against influenza A and B viruses, respiratory syncytial virus, parainfluenza virus, and a variety of exotic viral diseases (Gilbert et al., 1985; Hall et al., 1983; McIntosh et al., 1984; McCormick et al., 1986). Mycophenolic acid (MPA) currently is used as an immunosuppressant in kidney transplant patients as its orally bioavailable mycophenolate mofetil ester. In contrast to MPA, which inhibits IMP-D directly without requirement of metabolic conversion, ribavirin needs to be converted to its 5'-monophosphate derivative Rib-MP by adenosine kinase (Willis et al., 1978). Rib-MP inhibits IMP-D, thus decreasing the intracellular GTP and dGTP pools (Streeter et al., 1973). It has been shown that ribavirin potentiates the inhibitory effect of a variety of purine NRTIs, including ddA, ddI, ddG, 2',3'-dideoxy-2,6-diaminopurine riboside (ddDAPR), 3'-azido-ddDAPR (AzddDAPR), FddaraA, carbovir (CBV), and others (Baba et al., 1987; Balzarini et al., 1989, 1990; Hartman et al., 1991). Moreover, ribavirin also markedly enhances the anti-retrovirus activity of ddI, ddDAPR, and AzddDAPR in newborn NMRI mice infected with Moloney murine sarcoma virus (Balzarini et al., 1989, 1990).

A biochemical basis for the potentiating effect of ribavirin on the anti-HIV activity of the purine NRTIs has been proposed (Fig. 10) (Balzarini et al., 1991a). Ribavirin causes an increase in the levels of IMP, the presumed phosphate donor for the conversion of ddIno to dideoxyinosine monophosphate (ddIMP) by cytosolic 5'-nucleotidase (Johnson & Fridland, 1989). Consequently, ribavirin stimulates the conversion of ddI to its antivirally active metabolite 2',3'-dideoxyadenosine-5'-triphosphate (ddATP). In addition, ribavirin also causes a marked depletion of the guanine nucleotide pools. Thus, the accumulation of the IMP pools may result from (1) a direct inhibitory effect of ribavirin 5'-monophosphate on IMP dehydrogenase (which converts IMP to xanthosine monophosphate [XMP]) and (2) an indirect inhibition of adenylosuccinate synthetase by the decreased GTP and dGTP pools (since GTP is an obligatory cofactor in the conversion of IMP to succinyl AMP). However, it was demonstrated that GTP depletion plays a key role in the

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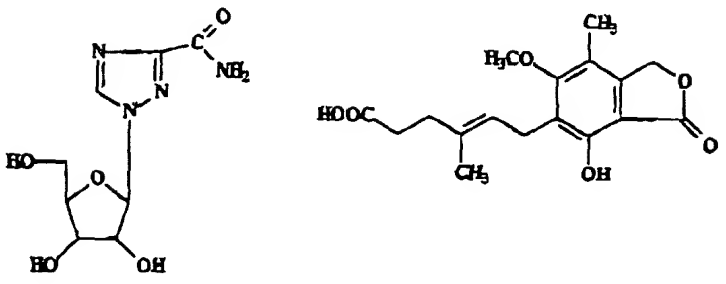

Ribavirin

Mycophenolic acid

Fig. 9. Structural formulae of the IMP dehydrogenase inhibitors ribavirin and MPA.

accumulation of IMP and the resulting higher rate of ddIno phosphorylation to ddIMP and, eventually, to ddATP (Balzarini et al., 1991a). This mechanism of potentiation of ddI metabolism is in agreement with the observations that guanosine and 2′-deoxyguanosine, but not 2′-deoxyadenosine, reverse (1) the stimulatory effect of ribavirin on the anti-HIV activity of ddIno and (2) the accumulation of endogenous IMP pools, as well as accumulation of IMP from exogenous hypoxanthine in ribavirin-treated cells. Other nucleoside analogues that potently inhibit IMP-D (e.g., 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide) have been shown to stimulate ddI activity upon combination of 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (Balzarini et al., 1991b).

Ribavirin does not necessarily have a beneficial effect on the anti-HIV-1 activity of other NRTIs. In sharp contrast with the purine NRTIs, ribavirin acts antagonistically when combined with pyrimidine NRTIs, such as AZT, d4T, and ddC (Vogt et al., 1987; Baba et al., 1987). This phenomenon can be explained by the increased dTTP and hence, dCTP pools afforded by ribavirin. This results not only in a less favorable competitive potential of 3′-azido-3′-deoxythymidine triphosphate (AZTTP) and d4TTP against HIV RT, but also in a more pronounced allosteric feed-back inhibition of dThd kinase by the increased dTTP levels, resulting in a lower ability of dThd kinase to convert AZT and d4T to their respective 5′-monophosphates.

It should be emphasized that IMP-D inhibitors may have a self-limiting effect on the potentiation of the anti-HIV activity of adenine and hypoxanthine NRTIs. Indeed, whereas increased (accumulating) intracellular IMP levels result in higher levels of ddIMP by the catalytic action of 5′-nucleotidase using IMP as the phosphate donor, the accumulating

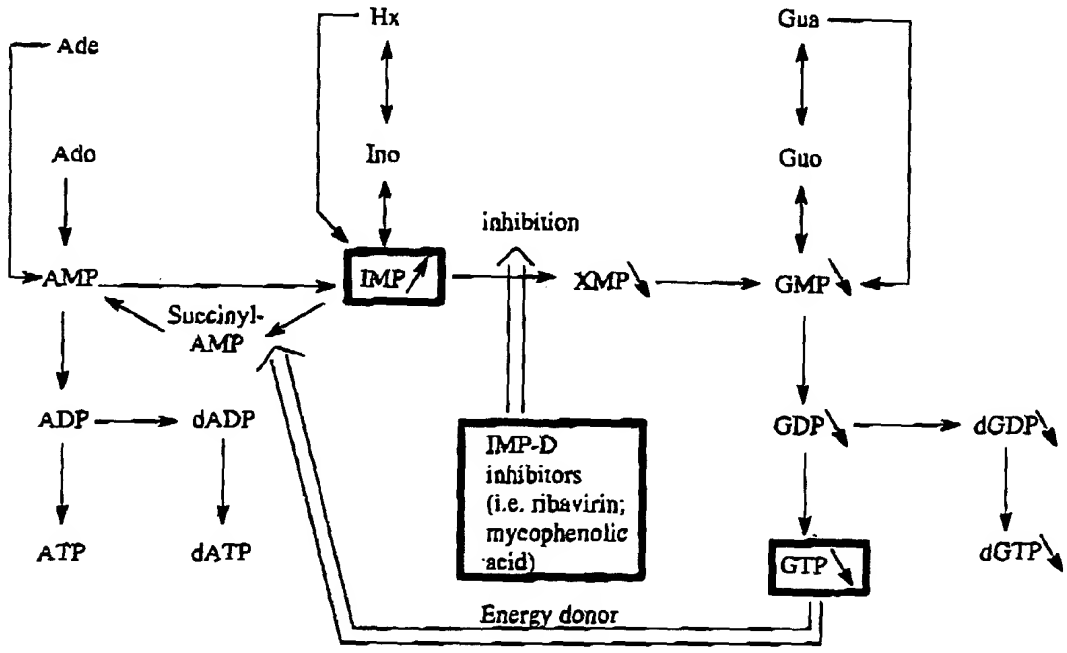

Fig. 10. Effect of IMP dehydrogenase inhibitors on the accumulation of IMP and depletion of GTP pools. The double open arrows represent inhibition of the IMP-D reaction (conversion of IMP to XMP) by IMP-D inhibitors and inhibition of the conversion of IMP to succinyl AMP by the lower GTP pools. GTP acts as an energy donor for the conversion of IMP to succinyl AMP. Both inhibitory effects result in increased IMP pool levels.

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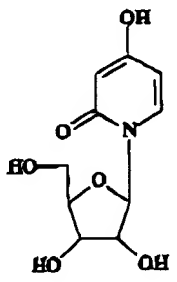
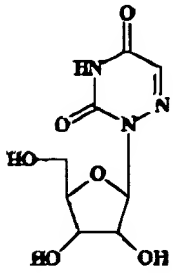
IMP levels will also compete with the higher ddIMP levels to become further converted to AMP and ddAMP, respectively, by two successive enzymatic steps (succinyl AMP lyase and succinyl AMP synthetase). In addition, due to depletion of GTP (and hence, dGTP) pools, the energy donor for the succinyl AMP lyase reaction becomes depleted in the cells, preventing efficient conversion of IMP, and also ddIMP, to AMP and ddAMP, respectively. Interestingly, the self-regulating limitation of this type of combination may not be in place if IMP-D inhibitors are combined with guanine NRTIs, such as 2′,3′-dideoxyguanosine and carbovir (Vince et al., 1989; Ahluwalia et al., 1990). Therefore, it can be anticipated that IMP-D inhibitors would better be combined with guanine NRTIs than adenine/hypoxanthine NRTIs. Moreover, the depletion of the dGTP pool will afford an additional advantage for combining IMP-D inhibitors with guanine NRTIs, due to the competitive advantage of the guanine NRTI-triphosphate against HIV RT in the presence of lower dGTP pools. In fact, it recently has been shown that mycophenolic acid has a pronounced synergistic antiherpetic activity when combined with anti-herpes guanine nucleoside analogues, such as acyclovir, ganciclovir, penciclovir, and lobucavir, not only in cell cultures, but also when evaluated in a small animal model (herpes simplex virus type 1-infected mice) (Neyts & De Clercq, 1998, 1999; Neyts et al., 1998a, 1998b).

6. Other potential targets in nucleotide metabolism that can be envisioned for combination with nucleoside reverse transcriptase inhibitors

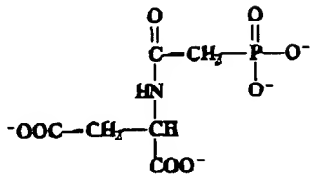
Following the same principles as for the combinations of RR inhibitors (i.e., HU) with ddI, and TS inhibitors (i.e., 5-FU,



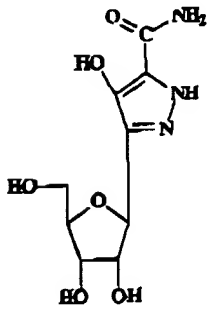
3-Deazauridine



6-Azauridine



N-(Phosphonacetyl)-L-aspartate (PALA)



Pyrazofurin

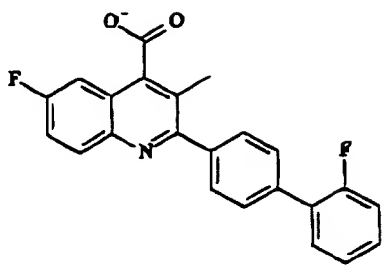


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Fig. 11. Structural formulas of 3-deazauridine, 6-azauridine, PALA, pyrazofurin, and brequinar.

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5-FdUrd. and MTX) with d4T and AZT, any combination of an antimetabolite drug with an NRTI can be beneficial for potentiation of the anti-HIV effect of the non-NRTIs, provided the antimetabolite depletes (selectively) the endogenous dNTP pool that is competing with the triphosphorylated NRTI for the virus-encoded RT. Thus, any modality that is able to deplete dTTP pools can be combined with thymine NRTIs, modalities that are able to deplete dATP pools can be combined with adenine NRTIs, modalities that are able to deplete dGTP pools can be combined with guanine NRTIs, and modalities that are able to deplete dCTP pools can be combined with cytosine NRTIs.

6-Azauridine and pyrazofurin, inhibiting orotidine 5'-monophosphate decarboxylase; *N*-(phosphonoacetyl)-L-aspartate (PALA), inhibiting aspartate transcarbamoylase; and 3-deazauridine, inhibiting CTP synthetase (Fig. 11) were shown to decrease endogenous dCTP pools and concomitantly increase ddC conversion to ddCTP (Balzarini et al., 1987) (Fig. 12). Also, pyrazofurin and brequinar, inhibiting dihydro-orotate dehydrogenase, were shown to increase d4TTP formation and to decrease endogenous dTTP levels (~50% of control at 1 and 5 μM, respectively) (Ahluwalia et al., 1996). Thus, such inhibitors of pyrimidine nucleotide biosynthesis may be advantageously combined with pyrimidine NRTIs, in particular, cytosine NRTIs, such as ddCyd and 3TC, or thymine NRTIs, such as d4T and AZT. Similarly, any antimetabolite drug that is able to decrease the purine dNTP pools and/or stimulate purine NRTI metabolism may be a suitable drug candidate to be combined with NRTIs currently included in HIV treatment regimens, such as ddI, ABC, or FddaraA.

7. Reflections and future perspectives

Various combinations of NRTIs with antimetabolites increase the anti-retroviral activity of NRTIs by decreasing the endogenous dNTP pools and/or by speeding up metabolism of the particular NRTI. However, the effects so far have not been dramatic enough to prefer such combinations over the combination therapies that are currently used in humans, the individual drugs of which are directed to different targets of the HIV replication cycle. Moreover, antimetabolite drugs usually afford their perturbing effect on dNTP pools and nucleotide metabolism at concentrations that are close to, if not exceeding, the toxicity threshold. Given the fact that anti-HIV compounds must be given for extended time periods that may exceed 1 year and longer, the therapeutic window for these types of compounds are expected to be rather narrow.

A number of other considerations, however, argue for the continuation of exploration of the potential of these tools in anti-retroviral combination therapy. In the particular case of HU, there exists a long experience of more than 30 years in the clinical management of this drug. It is diffusable in all body compartments and thus, able to potentiate the antiviral efficacy of NRTIs in some body compartments where the NRTI level is low or poorly active. Moreover, this drug is only mildly toxic, and the toxicity is reversible, disappear-

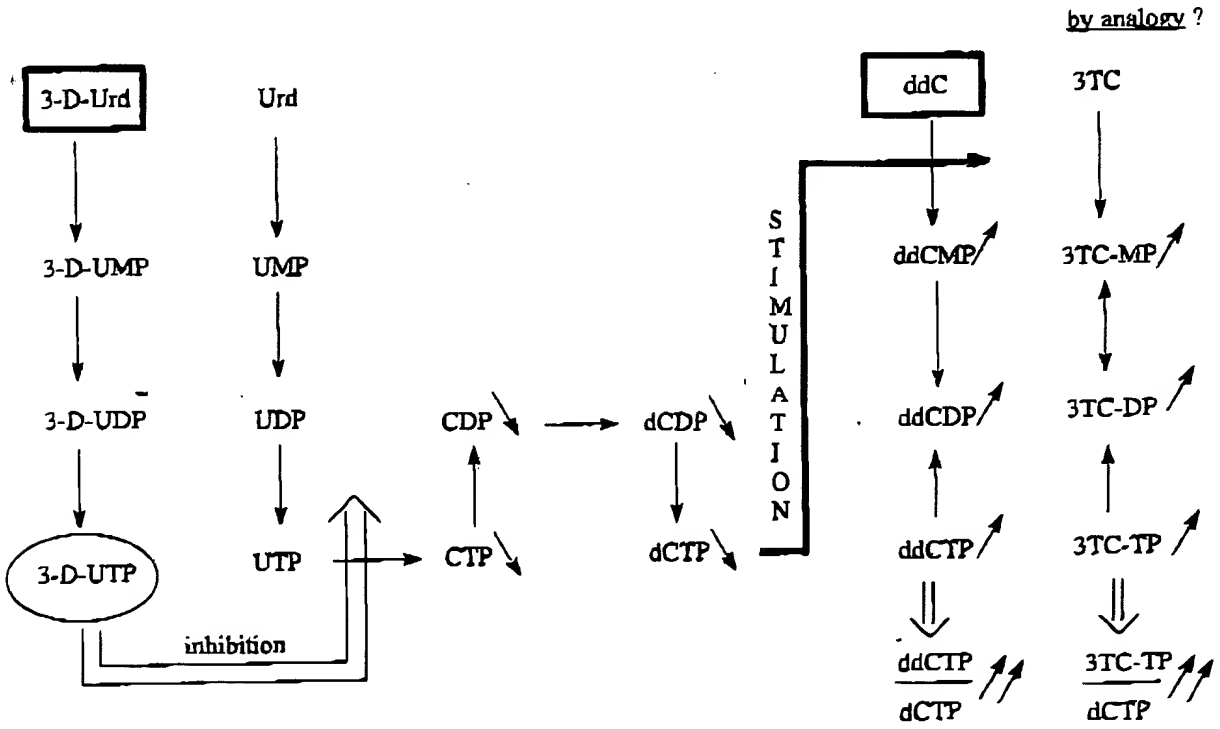

Fig. 12. Effect of the CTP synthetase inhibitor 3-deazauridine on the metabolism of pyrimidine nucleotides and the pyrimidine NRTI ddC. The closed black arrow represents somulation of 2'-deoxycytidine kinase (dCK) by the decreased dCTP pools. The open double arrow represents inhibition of CTP-S by 3-D-UTP, resulting in lower CTP and dCTP pools. The increased ddCTP and decreased dCTP levels result in increased ddCTP/dCTP ratios

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ing soon after its withdrawal. HU is also a relatively inexpensive drug, which may open perspectives as a valuable alternative for those patients who cannot afford the expensive triple or quadruple therapy that currently is promoted as the most efficient highly active anti-retroviral therapy. Indeed, if it turns out that one of the drugs that is a component of current drug mixtures can be replaced by HU or another antimetabolite drug preserving the antiviral potency of the original combined mixture, the introduction of antimetabolite drugs in current drug mixtures may become a feasible goal. Given the current problems associated with long-term administration of protease inhibitors (one of the drugs included in the majority of triple/quadruple drug mixtures), replacement of the protease inhibitor by an antimetabolite may help to potentiate one of the NRTIs present in the drug mixture.

An important issue is also the observation that antimetabolites may potentiate the NRTI in such a way that it may suppress NRTI-resistant virus strains, or may prevent or delay the appearance of NRTI-characteristic resistance mutations (see Johns & Gao, 1998; Balzarini, 1999). Since NRTI resistance afforded by a single mutation in the RT gene is usually not higher than 5- to 10-fold, it is reasonable to assume that such resistance mutations may be unlikely to appear at a similar rate as when the NRTI is not potentiated by the antimetabolite drug.

Last, but not least, it is unlikely that resistance of the virus will easily occur against the antimetabolite present in the drug combination, since this drug is directed against a cellular target and not a virus-specific enzyme or protein. This may represent a therapeutic edge over virus-specific drugs, given the emergence of drug-resistant virus strains upon prolonged exposure to the currently used drugs.

Acknowledgments

The author would like to thank Mrs. Inge Aerts and Mrs. Christiane Callebaut for excellent editorial assistance. Research on HIV by the author has been supported in part by the European Commission.

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